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(54) Title: NUCLEIC ACID–COBALAMIN COMPLEXES AND THEIR USE IN GENE THERAPY

(57) Abstract

Disclosed is a complex used to deliver nucleic acid to cells. In particular, disclosed are compositions and methods which use a nucleic acid encoding at least one protein, which nucleic acid is complexed with cobalamin. These compositions and methods utilize cobalamin receptor–mediated endocytosis for introduction of the protein–coding nucleic acid into cells. The compositions and methods allow for lower doses of nucleic acid to be used and reduce undesirable immunogenic effects.

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NUCLEIC ACID-COBALAMIN COMPLEYES AND THEIR USE IN GENE THERAPY

Field of the Invention

This invention relates to delivery of nucleic acid to cells for the production of proteins. In particular, it relates to the use of receptor mediated endocytosis for introduction of nucleic acid into cells so that lower doses of nucleic acid are required to produce a given effect and undesirable immunogenic effects are reduced.

Background of the Invention

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Currently, major gene transfer approaches employ either viral or nonviral vectors. Viral vector-directed gene transfer shows high gene transfer efficiency but is deficient in several areas. For example, some viral vectors randomly integrate DNA into host genomes, while others, such as adenoviral vectors, induce host inflammatory and immune responses, rendering these vectors ineffective in repeated application. Further, retroviral vectors require dividing cells for stable integration, making these vectors unsuitable for gene therapy of terminally differentiated cells. Similarly, for efficient transduction, adeno-associated virus prefers cells in the S-phase to cells in stationary culture. The high multiplicity of infection of adeno-associated virus for efficient transduction coupled with difficulty in obtaining virus preparations with high titer has limited the use of this virus as a routine gene therapy vector.[1]

Some of the problems associated with using these viral vectors can be circumvented using gene transfer agents, such as molecular conjugates prepared by chemically linking receptor ligands with polycations. Alternatively, molecular conjugates for receptor-mediated gene delivery can be prepared by chemically linking antibodies or fragments thereof with polycations. The polycations serve as carriers of DNA while the ligands target the receptors on cell surfaces. Upon binding to the receptors, the conjugates along with the DNA are internalized via receptor-mediated endocytosis. Receptor-mediated endocytosis has been used to introduce DNA into a variety of cells. Examples of these include an asialoosomucoid/polylysine conjugate complexed with a plasmid which utilizes the asialoglycoprotein receptor found on hepatocytes[4] and Fab fragments complexed with plasmid DNA which enter epithelial cells through the polymeric immunoglobulin receptor (pIgR).[5] Other ligands include mannose,[17] lactose,[21] insulin,[19] epidermal growth factor,[23] anti-thrombomodulin antibodies,[25] transferrin,[26]

gluconyl groups,[20] lectins,[16] folate[22] and fibroblast growth factor.[18, 24] The protein ligands can be difficult to prepare and can induce an unwanted immune response in the patient.

Cobalamin, also known as Vitamin B_{12} (VB_{12}), is a large molecule that cannot cross the epithelial barrier through simple or facilitated diffusion. Most fat soluble molecules, such as vitamins A, D, E and K, are generally absorbed in combination with fats, while the small water soluble vitamins, such as vitamin C, thiamine, riboflavin and folic acid are mainly absorbed by facilitated diffusion. Cobalamin, however, is absorbed through a mechanism that involves a number of transport proteins, which is markedly different than that of other vitamins. During the process of absorption, cobalamin is first bound by R-protein found in saliva. R-protein releases from cobalamin in the stomach where the cobalamin is then bound to Intrinsic Factor (IF) which forms a VB_{12}:IF complex. In the gut, this complex passes to the ileum where the complex binds in a calcium-dependent fashion to a specific intrinsic factor receptor (IFR) located on the apical membrane of the villous enterocyte. The complex is then internalized by the enterocyte via receptor-mediated endocytosis. Once inside the cell, the VB_{12} is released from the IF. In the blood, the transport protein for VB_{12} is Transcobalamin II (TcII) instead of IF.

Delivery of peptides and proteins across the intestinal epithelium using the VB_{12} uptake system has been described.[2, 3, 13, 14, 15] It has been shown that a conjugate of VB_{12} - D-Lys_6 analogue of luteinizing hormone-releasing hormone (LHRH) was active at stimulating ovulation in mice in an oral or intravenous dose, and that it is possible to link VB_{12} to erythropoietin (EPO) and to granulocyte-colony stimulating factor (G-CSF) in a manner to maintain the bioactivity of these substances *in vivo*. Covalent conjugation of therapeutically useful proteins to VB_{12} for oral co-administration with intrinsic factor is also known. [13] Further, antisense oligonucleotides have been functionalized with a VB_{12} cobamide coenzyme for their delivery to cells.[8, 10, 11] Use of VB_{12} as a carrier molecule coupled to a microparticle which contains nucleic acid has also been disclosed. [12]

Major problems associated with the use of nonviral (protein) and/or viral vectors are that a patient can develop a protein-related immune response with repeated use of these vectors or that vectors, e.g., polylysine, may trigger the complement cascade, when delivered via a non-oral route. Further, since nonviral vectors generally have low

efficiency as they are not taken up by specific receptors, high doses are required which can be toxic to the patient. Thus, there is a need for a vector which 1) may be used repeatedly without raising an immune response; 2) may be administered in high doses if needed; and 3) may be administered by a non-parenteral route.

Summary of the Invention

This invention is directed to complexes comprising a nucleic acid bound to cobalamin, methods of delivering nucleic acid into a cell and pharmaceutical compositions comprising a nucleic acid/cobalamin complex.

This invention is directed, in part, to the discovery that nucleic acid encoding a protein complexed with cobalamin may be introduced into a cell via cobalamin receptor-mediated endocytosis to deliver the nucleic acid into the cell so that it may encode a protein, which protein may be used locally, e.g., in the gut, or systemically. This may be a therapeutic protein, a nutritional protein or a protein used for research purposes. Further, since cobalamin is a required nutrient, the development of an immune response following repeated doses is unlikely.

In particular, this invention provides a complex and compositions comprising a nucleic acid, which nucleic acid encodes at least one protein, bound to cobalamin. The nucleic acid may be bound to the cobalamin with a suitable linker arm, especially one selected from the group consisting of polylysine and protamine. The complex may further comprise a cobalamin binding protein selected from the group consisting of intrinsic factor, R-proteins and transcobalamin II.

The nucleic acid encodes at least one protein, preferably a therapeutic protein, a nutritional protein or a marker protein. Marker proteins include, but are not limited to, β-galactosidase, secreted human placental alkaline phosphatase, chloramphenicol acetyl transferase (CAT), luciferase, green fluorescent proteins and derivatives thereof, blue fluorescent proteins and derivatives thereof and peptides or proteins conferring antibiotic resistance.

Another aspect of this invention is directed to a method for introducing nucleic acid into cells comprising administering to a subject an effective amount of a complex described above.

Finally, this invention is directed to a composition comprising the nucleic acid/cobalamin complex as described above and a pharmaceutically acceptable carrier.

The composition is adapted for administration via an oral, parenteral, mucosal, rectal or enteral route.

Detailed Description of the Invention

The present invention relates to a cobalamin construct useful for delivering nucleic acid into cells. In particular, the invention is directed to a nucleic acid encoding at least one protein, which nucleic acid is bound to a cobalamin molecule to form a cobalamin-nucleic acid construct. The binding of the nucleic acid to the cobalamin molecule may be directly or via a linker, preferably a polycation linker. Without being limited to any theory, it is believed that this construct delivers nucleic acid to cells via cobalamin receptor-mediated endocytosis.

The nucleic acid encodes at least one protein and may encode at least two proteins. The nucleic acid may be DNA or RNA. The encoded protein may be of therapeutic interest (therapeutic protein), may be a nutritional protein and/or may be a marker protein which may be used to indicate whether transfection has occurred. The encoded protein is preferably chloramphenicol acetyl transferase, β-galactosidase, secreted human placental alkaline phosphatase. Other encoded proteins include luciferase, green fluorescent proteins and derivatives thereof, blue fluorescent proteins and derivatives thereof and peptides or proteins conferring antibiotic resistance.

The cobalamin may preferably be cyanocobalamin or may be an analogue or derivative of cobalamin such as methylcobalamin, adenosylcobalamin or hydroxycobalamin. The derivative must bind nucleic acid and the bound form must be taken up by a cell which presents a cobalamin receptor.

The cobalamin-nucleic acid complex may comprise a linker arm, for example, polylysine, protamine, polyethyleneimine, DEAE dextran, polybrene, cationic lipids and ethidium salts. Preferred linkers are polycation linkers. The cobalamin-nucleic acid complex or the cobalamin-linker-nucleic acid complex may further be bound by a cobalamin binding protein, for example, intrinsic factor, R-proteins and transcobalamin II, or analogues thereof.

The compositions and methods of the present invention are useful clinically, nutritionally and/or as research tools for the study of various aspects of the activity of nucleic acids and proteins. They may be used in many ways *in vitro* or *in vivo*, for example, to study cellular or organ ontogeny, to study polarized secretion and protein secretory signals, to transfect cells which may be refractory to other methods, e.g.,

Caco-2 cells to determine transfection efficiency, to study the length of time the nucleic acid is expressed, and to determine whether a cell secretes the protein encoded by the nucleic acid introduced into the cell is secreted or is produced intracellularly.

The complex may be adapted for administration to a subject via a route selected from the group consisting of oral, parenteral, mucosal, rectal and enteral. Targeted delivery to cells which contain cobalamin receptors, for example, upper gastrointestinal tract cells, lower gastrointestinal tract cells and liver cells, may be accomplished either by direct administration to the cells, e.g., by instillation or deposition in the intestine, or by the use of various delivery systems which provide targeted delivery, e.g., enteric coated dosage forms, colonic dosage forms, delivery directed to the liver, etc.

The effective amount of the nucleic acid/cobalamin complex delivered to a subject is that amount which results in satisfactory protein production levels, and is dependent upon the protein, subject, goal of administration (e.g., testing, treating or prevention), route of administration, condition treated, and the like.

The description of this invention is best understood in light of the following terms, which, for the purposes of this application, have the following meaning:

A. Definitions

"Cobalamin" as used herein refers to the compound commonly known as either Vitamin B_{12} (VB_{12}) or cyanocobalamin. "Cobalamin derivatives" refers to hydroxycobalamin, adenosylcobalamin, methylcobalamin, aquocobalamin, 5-O-methylbenzylcobalamin, as well as the desdimethyl, monoethylamide and the methylamide analogues of all of the above. Also included are the various analogues and homologues of cobamide such as coenzyme B_{12} and 5'- deoxyadenosylcobalamin. Other analogues include chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanocobalamin, benzimidazole derivatives such as 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole, as well as adenosylcyanocobalamin, cobalamin lactone, cobalaminlactam and the anilide, ethylamide, monocarbozylic and dicarboxylic acid derivatives of VB_{12} or its analogues. Other analogues include analogues which replace the D-ribose moiety of the nucleotide loop with an oligomethylene group or a trimethylene analogue containing imidazole instead of 5,6-dimethylbenzimidazole, as described in Ishida et al., Arch. Microbiol. (1994) 161:293-299, which is incorporated by reference in its entirety. Additionally included are analogues where the cobalt is replaced by zinc or nickel, or analogues where the corrin

ring, or any part of the cobalamin molecule, has one or more substituents. It is understood that analogues which are useful in the present invention are those which bind to the nucleic acid and/or a linker arm, and retain the capacity to bind to the cobalamin-binding proteins, such as Intrinsic Factor (IF). Assays to determine such binding are known to those of skill in the art. (See, for example, Habberfield, et al., *Vitamin B12-mediated uptake of erythropoietin and granulocyte colony stimulating factor in vitro and in vivo*, International Journal of Pharmaceutics 145:1-8 (1996).) Preferably, cyanocobalamin, hydroxycobalamin, adenosylcobalamin or methylcobalamin is used in this invention.

"Cobalamin-binding proteins" or "cobalamin-binding transport proteins" include intrinsic factor (IF), R-proteins such as transcobalamin I and transcobalamin III, and transcobalamin II (TCII) and analogues thereof which bind to and transport cobalamin into a cell. Intrinsic factor, a glycoprotein, is secreted by the stomach, while transcobalamin II, a polypeptide, is present in plasma. The R-proteins, a family of glycoproteins, are found in plasma, granulocytes and several glandular secretions. These proteins bind to cobalamin in a macromolecular complex that involves a binding site on the protein which allows the cobalamin-binding protein/cobalamin complex to enter a cell via cobalamin receptor mediated endocytosis. Receptors for intrinsic factor are found on microvillous membranes of ileal absorptive cells, e.g., ileal mucosal cells, (ileal enterocytes). A wide variety of cells, including dividing cells, have receptors for transcobalamin II, and receptors specific for R-proteins are present on hepatocytes.

"Linker" refers to a structure which is covalently or non-covalently bound to cobalamin, or a derivative thereof, and which binds nucleic acid, permits the transport of cobalamin into the cell and does not interfere with the coding ability of the nucleic acid once the complex has entered the cell. Examples of linker arms suitable for use in this invention include polylysine, protamine, polyethyleneimine (see Boussif et al., Proc. Natl. Acad. Sci., (1995) 92:7297-7301), DEAE dextran, polybrene, cationic lipids, and ethidium salts. Avidin or streptavidin-biotin may be used as the linker structure, e.g., a nucleic acid-avidin linked to biotinylated VB_{12}. Additionally, nucleic acid binding proteins, transcription factors, histones and other chromatin associated proteins may also be used as linkers. Cationic linkers are preferred.

"Cobalamin-linker conjugate," also referred to as "conjugate," refers to cobalamin or a cobalamin derivative conjugated, covalently or non-covalently, to a linker, for example polylysine or protamine.

"Cobalamin-nucleic acid complex", also referred to as "complex" refers to nucleic acid bound to a cobalamin molecule. This complex may include a linker, where the linker is bound to the cobalamin and binds the nucleic acid. This complex is capable of being bound to a cobalamin binding protein such as intrinsic factor, R-protein or transcobalamin II. The cobalamin-nucleic acid complex bound to a cobalamin-binding protein is capable of utilizing a cobalamin receptor to enter a cell via receptor-mediated endocytosis.

"Nucleic acid" (NA) or "nucleic acid construct" refers to unbranched (linear or circular) chains of nucleotides which encode a protein or peptide. This includes deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The nucleic acid construct may be in the form of a plasmid. The nucleic acid may include promoter, leader, signal, polyadenalyation or intron sequences, locus control regions, markers and the like. The nucleic acid may utilize bacterial or mitochondrial codons. Nucleic acid containing modified, derivatized or non-naturally occurring nucleotides are also included within this definition.

A "protein" refers to polymers which comprise amino acid residues bound together by amide linkages (CONH). Both naturally-derived or recombinantly produced moieties are included in this definition. This includes post-translationally modified forms, e.g., glycosylated proteins and peptides. A "nutritional protein" is a protein or peptide which provides nutrition to a cell or organism. A "therapeutic protein" is a protein or peptide which may provide a pharmacologic effect, generally beneficial, to an organism. This effect may be localized in the gastrointestinal tract or may be systemic. Examples of therapeutic proteins that provide localized therapy to the gastrointestinal tract include, but are not limited to, pancreatic enzymes, lactase, cytokines, interleukin-1 receptor antagonist, tumor necrosis factor receptor, recombinant antibodies and antibody fragments, tumor suppressor proteins, cytotoxic proteins and the like.

The nucleic acid of this invention encodes at least one protein, which may or may not be of therapeutic interest. Useful non-therapeutic proteins include marker proteins. A "marker protein" is any protein which may be used, often as a research tool, to determine whether transfection has occurred or to monitor the length of time the nucleic

acid remains active in the cell. Any protein for which expression can be determined may be used as a marker protein, even though the protein may also have a therapeutic use. Suitable marker proteins can include, but are not limited to, luciferase, β-galactosidase, secreted human placental alkaline phosphatase (SEAP), CAT, blue and green fluorescent proteins and proteins conferring antibiotic resistance. Encoded therapeutic peptides and proteins may include, but are not limited to insulin (for the treatment of diabetes), α1-antitrypsin (for α1-antitrypsin deficiency); Factor VIII (for hemophilia), Factor IX and other coagulation factors (for bleeding disorders); growth hormones (such as human growth hormone for growth disorders); other peptide hormones; growth factors (such as fibroblast growth factor (FGF), platelet derived growth factor (PDGF)); pituitary hormones (such as adrenal cortical stimulating hormone (ACTH), thyroid stimulating hormone (TSH)); lymphokines and cytokines for systemic therapy; γ-interferon (for granulomatous disease of childhood); α-interferon (for leukemia and chronic active hepatitis); erythropoietin; other hematologic growth factors (for chronic renal failure and other marrow suppressive disorders); tissue plasminogen activator (TPA) (for prevention of thrombosis in the pulmonary coronary arteries); CD4 (for human immunodeficiency virus (HIV) infection); proteins for other hereditary disorders such as cerebrosidase deficiency and adenosine deaminase deficiency; receptor agonists or antagonists (such as for the control of systemic hypertension, interleukin-1 receptor antagonist for septic shock, rheumatoid arthritis and other disorders); binding proteins for cytokines, lymphokines and hormones (such as tumor necrosis factor (TNF)), chymotrypsin, trypsinogen, lactate dehydrogenase, clotting factors, enzymes, immune response stimulators, interferons, immunoglobulins, interleukins, peptides, somatostatin, somatotropin analogues, somatomedin-C, Gonadotropic releasing hormone, follicle stimulating hormone, luteinizing hormone, LHRH, LHRH analogues such as leuprolide, nafarelin and goserelin, LHRH agonists and antagonists, growth hormone releasing factor, calcitonin, colchicine, gonadotropins such as chorionic gonadotropin, oxytocin, octreotide, somatotropin plus an amino acid, vasopressin, adrenocorticotrophic hormone, epidermal growth factor, prolactin, somatotropin plus a protein, cosyntropin, lypressin, polypeptides such as thyrotropin releasing hormone, thyroid stimulation hormone, secretin, pancreozymin, enkephalin, glucagon, endocrine agents secreted internally and distributed by way of the bloodstream, and the like. Further agents that may be delivered include peptide

hormones, adrenal cortical stimulating hormone, thyroid stimulating hormone, and other pituitary hormones, interferon α, β, and γ, consensus interferon, erythropoietin, growth factors such as GCSF, GM-CSF, insulin-like growth factor 1, CF4, dDAVP, pancreatic enzymes, lactase, interleukin-2, tumor suppresser proteins, cytotoxic proteins, retroviruses and other viruses, viral proteins, antibodies, recombinant antibodies, antibody fragments and the like. Tumor antigens, allergens and bacterial or viral proteins, which induce an immune response or which results in tolerization are also examples of therapeutic proteins.

"Pharmaceutically acceptable carrier" refers to biologically acceptable carriers.

"Transfection enhancer" refers to compounds which enhance the efficiency of transduction of non-viral vectors. They may be given therapeutically. An example of a transfection enhancer useful for oral delivery of nucleic acid constructs is chloroquine.

B. Pharmacology

The methods of this invention are achieved by using pharmaceutical compositions comprising a cobalamin-nucleic acid complex which binds in concert with a transport protein to a cobalamin receptor and is endocytosed into a cell, thereby allowing expression of the nucleic acid in the cell. Preferably, the cell is an epithelial cell and more preferably, an enterocyte and even more preferably, an ileal absorptive cell.

It is understood that the compounds of this invention may be made into pharmaceutical formulations for delivery to a patient. The cobalamin-nucleic acid complex may be administered alone, with a cobalamin-binding protein bound to the cobalamin-nucleic acid complex, or the complex may be co-administered with biologically active binding proteins, such as intrinsic factor. When administered alone, the cobalamin-binding proteins normally found in the patient's stomach, plasma, granulocytes or glandular secretions are utilized to bind to the cobalamin-nucleic acid complex so that cobalamin receptor-mediated endocytosis can occur which transports the nucleic acid into the cell.

When used for oral administration and delivery to the small intestine, which is preferred, the cobalamin-nucleic acid complex compositions may be formulated in a variety of ways. The cobalamin-nucleic acid complex or cobalamin-nucleic acid/cobalamin binding protein complex may be administered as a solid formulation. Solid formulations may contain a pharmaceutically inert carrier, including conventional

carriers such as lactose, starch, dextrin or magnesium stearate. These are conveniently presented in a tablet or capsule form. Preferably, the formulation is encapsulated in an enteric coated capsule, e.g., a coating which remains intact in the stomach, but dissolves and releases the contents of the capsule once it arrives in the small intestine. In the delivery of a bolus dose to the intestine, the total delay time prior to the delivery of the cobalamin-nucleic acid construct is usually in the range of less than about 1 hour, usually within 45 minutes, and preferably in less than about 30 minutes for delivery of the complex to the small intestine. One example is the PULSINCAP ™ which comprises an impermeable capsule and a hydrogel plug. The plug is designed to swell and dislodge from the devise after a particular time period, thereby delivering its contents in a bolus following a predetermined delay. See, for example, International Patent Application WO 96/40081, published 19 December 1996, which is incorporated by reference in its entirety. See also, Remington's Pharmaceutical Sciences, 18th edition, 1990, especially Chapter 89, for other oral pharmaceutical preparations which can be used in this invention.

Alternatively, the formulation can be a liquid formulation or semisolid form for oral dosage. Liquid formulations include a pharmaceutically inert carrier such as water, saline or other pharmaceutically acceptable liquids for oral administration. The liquid is compatible with the cobalamin-nucleic acid complex or the cobalamin-nucleic acid/cobalamin-binding protein complex. A nasogastric tube may also be used to deliver the liquid compositions directly to the stomach. The use of such liquids is well known to those skilled in the art. Semi-solid formulations may also be used. These may include the cobalamin-nucleic acid complex or cobalamin-nucleic acid/cobalamin-binding protein complex incorporated into gelatin, such as flavored JELLO®, and other soft foods such as applesauce, pudding or ice cream.

The compounds of this invention may also be administered directly to the ileum through surgical access.

For hereditary and acquired disorders of the gastrointestinal tract, oral administration of the cobalamin-nucleic acid complex provides a means of administering proteins to the cells of the epithelium. This method provides a means of delivery of nucleic acid encoding proteins which are secreted into the lumen, interstitially or systemically, such as pancreatic enzymes, lactase (for lactose intolerance), etc. Examples of systems which may be used to deliver gene constructs to the intestine are

disclosed, for example, in Henning, S.J. et al., *Gene therapy using the intestine*, WO93/19660, published 14 October 1993.

When parenteral, e.g., intravenous, administration is desired, the cobalamin-nucleic acid complex or cobalamin-nucleic acid/cobalamin binding protein complex may be suspended in a pharmaceutically acceptable carrier, such as water, saline, phosphate buffered saline, perfluorodecalin, etc. See Remington's Pharmaceutical Sciences, 18th edition, 1990 for suitable carriers and formulations for parenteral administration.

Doses are selected to provide an effective amount of cobalamin-nucleic acid to cells. Depending upon the purpose, this can be a detected amount, an amount that causes or attenuates an immune response to the coded protein, or an amount effective to ameliorate or prevent a disease condition or disorder. These compositions may be formulated in a unit dose form or in multiple or subunit doses. When oral administration is desired, the clinician should keep in mind that enterocytes have about a three day turnover in the ileum. The dose level and schedule of administration may vary depending upon the particular condition treated, the age, weight and overall health of the patient. Determination of this dosage is well within the skill of the clinician or researcher. (See, e.g., Goodman and Gilman, *The Pharmacological Basis of Therapeutics*, 7th Ed., the disclosure of which is incorporated herein by reference.)

The cobalamin-nucleic acid complex compositions of this invention may be administered to animals in the manner and formulations described above. These include mammals such as humans, cattle, pigs, dogs, cats, horses, sheep, goats and rodents; avians such as chicken, turkey, duck and geese; and any other animal which has cobalamin receptors.

C. Methodology

The abbreviations used throughout have the following meanings unless otherwise indicated:

mg	=	milligram
µg	=	microgram
ml	=	milliliter
µl	=	microliter
mm	=	millimeter
cm	=	centimeter
mM	=	millimolar
M	=	molar
C	=	Celsius

MW	=	molecular weight
h	=	hour
NA	=	nucleic acid
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
nm	=	nanometer
PBS	=	phosphate buffered saline
BSA	=	bovine serum albumin

PREPARATION OF CYANOCOBALAMIN-POLYLYSINE CONJUGATE

Cobalamin can be bound to nucleic acid either directly (covalently) or via an attached linker arm.

Cyanocobalamin monocarboxylic acids were prepared and purified as described in Anton et al., J. Amer. Chem. Soc. (1980) 102:2215-2219. Forty-five mg of the cyanocobalamin carboxylic acid (CNCb/IICOOH) was reacted with 50 mg hydroxybenzotriazole, 114 mg 1-ethyl, 3(3'dimethylaminopropyl)carbodiimide and 300 mg polylysine (MW 15,000-30,000, Sigma Chemical Co., St. Louis, MO) in a final volume of 15 ml of water. The pH was adjusted to 6.35 with NaOH and the reaction was allowed to proceed for 6-48 hours at room temperature.

The reaction mixture was then applied to a 43 X 3 cm Sephadex G-25 column. The high molecular weight fraction, containing both the derivatized and intact polylysine, was collected.

This fraction may be further purified by passing over an affinity column (e.g., a 2.1 mm by 5 cm or 1.0 mm by 3 cm Poros perfusion column (PerSeptive Biosystems)) prepared with immobilized R-protein (non-intrinsic factor, Sigma Chemical Co.) The cobalamin-polylysine conjugate is retained bound to the R-protein on the column and the underivatized polylysine passes through. The column is then further washed with the running buffer (buffered isotonic HEPES solution) to remove residual underivatized polylysine and other reaction by-products. Finally, the purified cobalamin-polylysine conjugate is eluted from the column by applying a mild organic modifier (such as MeOH or ACN) or by changing the pH of the running buffer.

The eluted cobalamin-polylysine conjugate was then extensively dialyzed. The concentration of polylysine was determined by standard protein assay procedures (e.g., BCA kit using manufactures instructions (Pierce)). The concentration of cobalamin was determined by absorbance at 550 nm. A 1% solution of cobalamin has an absorbance

of 64 at this wavelength. From the concentration of these two species, the average number of cobalamin molecules per polylysine was determined and found to be 3.7 cobalamin molecules per polylysine.

Polylysine is preferred as a linker, however, it is understood that other linkers may be conjugated to cobalamin including protamine, nucleic acid binding proteins, transcription factors, histones and the like. Useful linkers are those able to bind to or associate with cobalamin and nucleic acid without preventing either binding to cobalamin receptors or interfering with the ability of the nucleic acid to encode a protein.

Further, it is understood that the above stoichiometry of reagents may be varied to alter the number of cobalamin residues bound to each polylysine molecule as needed, and that the length of the polylysine chain may be varied to optimize nucleic acid binding and cellular uptake of the nucleic acid of interest. This process is well within the skill of the art.

DETERMINATION OF NUCLEIC ACID BINDING CAPACITY

To verify that nucleic acid binds to the cobalamin-polylysine conjugate, purified plasmid nucleic acid was mixed with the conjugate in varying charge ratios. For experimental purposes, the polylysine was assumed to have a charge of 4.1×10^{15} positive charges per microgram, while the nucleic acid was assumed to have a charge of 9.1×10^{14} negative charges per microgram. Charge ratios ranged from 1:20 to 20:1 nucleic acid per polylysine. The mixtures were then subjected to agarose gel electrophoresis, with unbound nucleic acid simultaneously run on the gel as a control.

Following electrophoresis, the nucleic acid was visualized by staining the gel with ethidium bromide (Sigma). The unbound nucleic acid remaining in the mixture sample was observed to run at a similar rate as the control nucleic acid. The nucleic acid that was bound to the cobalamin-polylysine conjugate was unable to enter the gel and was retained in the well. The nucleic acid was found to bind quantitatively at charge ratios of 1:1 to 1:20.

EVALUATION OF BINDING OF COBALAMIN-BINDING PROTEINS TO THE COBALAMIN-NUCLEIC ACID COMPLEX

The binding of cobalamin-binding proteins to the cobalamin-nucleic acid complex may be evaluated as follows. Cobalamin (alone) and cobalamin-nucleic acid complexes are diluted in six-tenfold dilutions in intrinsic factor buffer (1 mg/ml BSA (VB_{12} and IF deficient, Sigma) in 0.1 M phosphate buffer pH7.5). To 225 µl of the IF buffer is added 25 µl of the above dilutions. $Co^{57}VB_{12}$ (0.25 ml, 0.25 ng in IF buffer) is then added to

each sample. Porcine IF (0.25 ml; 1U/ml in IF buffer) is then added and the material allowed to incubate at room temperature for 20 minutes. BSA-coated charcoal (0.25 ml; 0.5% BSA (B_{12} and IF free) plus 2.5% charcoal) is added to each sample, vortexed and centrifuged. The supernatant from each sample is then counted on a gamma counter set for counting Co^{57}. Results are determined as a percentage inhibition of the Co^{57}-VB_{12} binding.[12]

Alternatively, the binding capacity of the cobalamin-nucleic acid complex to the cobalamin-binding proteins, e.g., intrinsic factor, R-proteins and transcobalamin II (each available from Sigma), is assessed by the ability of the complex to compete with underivatized cobalamin. The binding capacity can be determined by using a number of commercially available kits, such as MAGIC® LITE VITAMIN B_{12} ASSAY, available from Ciba Corning Diagnostics Corporation. This assay is a competitive chemiluminescence receptor assay. Other commercially available assays which may be used to assess the binding capacity include MAGIC® Vitamin B_{12} /Folate Radioassay (Ciba Corning Diagnostics Corporation) and QUANTAPHASE II® B_{12}/Folate Radioassay (BioRad, Hercules, California).

The binding affinity of the cobalamin-nucleic acid complex is compared with that of the control, uncomplexed cobalamin. The binding affinity for complexes preferred for use in this invention is at least 1% of control binding affinity.

EVALUATION OF TRANSFECTION

1. *IN VITRO* EVALUATION

To evaluate transfection of the cobalamin-polylysine-nucleic acid complex, Caco-2 cells (Fogh, J. Natl. Cancer Inst. (1977) 59:224-226) or other cell lines bearing cobalamin receptors, e.g., HepG2 cells (Hall et al., J. Cell Physiol. (1985) 124(3):507-515), opossum epithelial cells OK (Ramanujam et al., J. Biol Chem (1991) 266(20):13135-13140), porcine kidney LLC-PK1 (Ramanujam), human erythroleukemic cell line K562 and HL60 (Amagasaki et al, Blood (1990) 76(7):1380-1386), Murine lymphoma cell line BW5147 (McLean et al., Blood (1997) 89(1):235-242), and human colonic carcinoma cell HT29 (Gueant et al., FEBS Lett (1992) 297(3):229-232) are used. The nucleic acid is a plasmid nucleic acid encoding a marker protein such as chloramphenicol acetyl transferase (CAT). Alternatively, other linkers may also be used. The cobalamin-polylysine-nucleic acid complex is mixed at various charge ratios as described above.

Various concentrations of the complex, either in appropriate culture medium or in PBS containing 1.7mM $CaCl_2$, is applied to subconfluent or confluent differentiated Caco-2 cells in culture. One to 24 hours later, the complex is removed and culture medium is applied. Twenty-four to 96 hours after the initial application of complex, the cells are evaluated for the expression of the marker protein, for example, CAT.

A variety of standard techniques can be used detect the expression of the marker protein. For example, in the case of CAT, histochemical staining can be used to determine the efficiency of transduction. The protein product can be quantified in cell-free extracts by ELISA (CAT ELISA kit, Boehringer-Manneheim). Histochemical staining for CAT is accomplished by fixing cells using 50% methanol, 50% acetone for 2 minutes. Washed cells are incubated with rabbit anti-CAT antibody (5'-3') diluted 1:150 in 10% fetal bovine serum, PBS for 1 hour at room temperature. Cells are then washed in PBS and incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (Caltag) diluted 1:150 in 10% fetal bovine serum, PBS for 1 hour at room temperature. Cells are washed and incubated with the colorigenic HRP substrate O-dianisidine in 27% hydrogen peroxide. Color development is allowed to proceed 30 minutes to overnight. Cells expressing CAT are scored by microscopic examination.

The concentration of the complex, the amount of time the complex is left on the cells, the charge ratio of the cobalamin conjugate to nucleic acid, the polylysine chain length and the stoichiometry of the cobalamin to polylysine may be varied to optimize the efficiency of transfection.

2. *IN VIVO* EVALUATION

Transfection of intestinal cells *in vivo* may be evaluated using the ileal loop model. Rats, and other species such as rabbits, may be used in this model.

Twenty 300 to 350 g Sprague Dawley rats (Charles River) are anesthetized. Using aseptic technique, a midline abdominal incision is made and the ileum is located. The intestinal contents are gently removed using warmed saline and the cobalamin-polylysine-nucleic acid complex is injected. Alternatively, Intrinsic Factor (Sigma) bound to the cobalamin-polylysine-nucleic acid complex may also be used. The treated segment is marked at each end using sutures to allow subsequent relocation. Soft ligatures may be placed at each end of the flushed segment prior to injection of the complex. In these experiments, the ligated, treated segment is returned to the

abdominal cavity for two hours where it is kept moist. The ligatures are then removed and the animal is closed and allowed to recover.

To evaluate the transfection of intestinal cells, 5 rats are euthanized at 24, 48 and 72 hours. Treated tissues are excised and washed twice in cold PBS. Protein expression is determined, for example, using the β-gal ELISA kit (Boehringer-Manneheim), when β-galactosidase is the encoded protein. Tissues are rinsed in the lysis buffer provided (4mM Pefablock, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamidine, 1μg/ml pepstatin, 5 μg/ml aprotinin (Sigma), 0.5 mM EDTA and 0.5 mM dithiothreitol). Tissues are homogenized in 0.5-1 ml lysis buffer using a Brinkman polytron. Following homogenization, extracts are centrifuged at 14,000 rpm for ten minutes. The supernatants are quick frozen and stored at -70° C. The total protein concentration is determined using a BCA kit (Pierce) and adjusted to 25 mg/ml by diluting in lysis buffer containing protease inhibitors. β -galactosidase concentrations are determined by ELISA following manufacturer's instructions.

For histochemical staining of β-galactosidase in intestinal tissue, intestinal segments are rinsed thoroughly in cold PBS and frozen in Tissue-Tek O.C.T. compound by immersing in an isopentane bath equilibrated with ethanol/dry ice. Sections, eight to ten μm long, are prepared and immediately fixed for 10 minutes in 0.5% glutaraldehyde, washed in PBS and immersed overnight in PBS containing 5mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal, Sigma). The sections are viewed under a microscope. The appearance of a blue stain indicate transfection has occurred, and no blue staining indicate that transfection has not occurred. Endogenous β-galactosidase activity does not interfere with this assay when performed under these conditions. See Foreman, P. et al, *Adenoviral Gene Transfer to the Intestine*, Human Gene Therapy, submitted for publication 3/9/98.

Alternatively, the gene encoding the secreted form of human placental alkaline phosphatase (SEAP) may also be used to quantify transfection. This enzyme is readily distinguished from other serum alkaline phosphatases in rats because it is resistant to the inhibitor L-homoarginine and retains its activity after incubation at 65°C for 30 minutes.

Serum samples are obtained from rat tail veins prior to administration of the complexed DNA and at various times (e.g., 24 to 72 hours) after treatment. Following

manufacturer's instruction, 10-25 µl of serum are diluted in 75-200 dilution buffer provided in the kit. The presence of secreted human placental alkaline phosphatase is determined using the Phospha-Light kit (Tropix, Inc.), a chemiluminescent reporter gene assay.

It will be appreciated by those skilled in the art that this invention can be embodied in other specific forms without departing from the spirit or essential character thereof. The presently disclosed embodiments are therefore considered in all respects to be illustrative and not restrictive. The scope of this invention is indicated by the appended claims and all changes which come within the meaning and range of equivalents thereof are intended to be embraced therein.

The disclosure of each patent, patent application or reference cited in this application is hereby incorporated by reference in its entirety.

What is claimed is:

1. A complex comprising a nucleic acid, said nucleic acid encoding at least one protein, which nucleic acid is bound to cobalamin.

2. The complex of claim 1 further comprising a linker arm which links the nucleic acid to the cobalamin.

3. The complex of claim 2 wherein the linker arm is a polycation.

4. The complex of claim 2 wherein the linker arm is selected from the group consisting of polylysine, protamine and polyethyleneimine.

5. The complex of claim 1 further comprising a cobalamin binding protein.

6. The complex of claim 5 wherein the cobalamin binding protein is selected from the group consisting of intrinsic factor, R-proteins and transcobalamin II.

7. The complex of claim 1 wherein the at least one encoded protein is selected from the group consisting of a therapeutic protein, a nutritional protein and a marker protein.

8. The complex of claim 1 wherein the nucleic acid encodes at least two proteins.

9. The complex of claim 8 wherein the nucleic acid encodes at least two proteins selected from the group consisting of a therapeutic protein, a nutritional protein, and a marker protein.

10. The complex of claim 1 wherein the encoded protein is selected from the group consisting of β-galactosidase, secreted human placental alkaline phosphatase, chloramphenicol acetyl transferase, luciferase, green fluorescent proteins, and blue fluorescent proteins.

11. The complex of claim 1 wherein said cobalamin is a cobalamin derivative.

12. The complex of claim 11 wherein said cobalamin derivative is selected from the group consisting of methylcobalamin, adenosylcobalamin and hydroxycobalamin.

13. The complex of claim 10 wherein said nucleic acid is RNA or DNA.

14. A method for introducing nucleic acid into cells comprising providing to the cells an effective amount of the complex of claim 1.

15. The method of claim 14 wherein the complex is administered to an animal.

16. The method of claim 14 wherein the is selected from the group consisting of avians and mammals.

17. The method of claim 16 wherein the mammal is a human.

18. The method of claim 14 wherein the complex provided to the cells is by administration via a route selected from the group consisting of oral, parenteral, mucosal, rectal and enteral.

19. The method of claim 14 further comprising a transfection enhancer.

20. The method of claim 19 wherein the complex is provided by oral administration and the transfection enhancer is chloroquine.

21. A composition comprising the complex of claim 1 and a pharmaceutically acceptable carrier.

22. The composition of claim 21 adapted for administration selected from the group consisting of oral, parenteral, mucosal, rectal and enteral.

23. The composition of claim 22 wherein the pharmaceutically acceptable carrier is selected from the group consisting of water, saline, perfluorodecalin and phosphate buffered saline.

24. A delivery system which targets cells bearing cobalamin receptors comprising the complex of claim 1.

25. The delivery system of claim 24 wherein the delivery system is selected to deliver the complex of claim 1 to cells selected from the group consisting of upper gastrointestinal tract cells, lower gastrointestinal tract cells and liver cells.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/12236

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUY M ET AL: "Evaluation of coupling of cobalamin to antisense oligonucleotides by thin-layer and reversed-phase liquid chromatography" JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS, vol. 706, no. 1, 27 February 1998 (1998-02-27), page 149-156 XP004110841 ISSN: 0378-4347 * abstract; p.149, 2nd col.,l.10-p.150, 1st col., l.30; p.p.155-156, discussion * ---	1-25
Y	WO 93 07883 A (ISIS PHARMACEUTICALS INC) 29 April 1993 (1993-04-29) claim 39 --- -/--	1-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 October 1999	02/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Uiber, P

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/12236

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 17167 A (BIOTECH AUSTRALIA PTY LTD) 15 October 1992 (1992-10-15) claims 1,4,5	1-25
A	EP 0 220 030 A (BIOTECH AUSTRALIA PTY LTD) 29 April 1987 (1987-04-29) cited in the application claims 1-28	1-25

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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